

**METHOD OF ISOLATING AND CULTURING MESENCHYMAL
STEM CELL DERIVED FROM CRYOPRESERVED UMBILICAL CORD
BLOOD**

5 **Technical Field**

The present invention relates to a method of isolating and culturing a mesenchymal stem cell using umbilical cord blood that is most ideal for a cell therapy. More particularly, it pertains to a reproducible method of isolating and culturing a mesenchymal stem cell using umbilical cord blood cryoperserved at -196°C .

Background Art

15 Mesenchymal stem cells refer to primitive cells that are able to differentiate into bone, cartilage, adipose tissue, nerve and muscle, etc., and are known as being contained in a large amount in bone marrow. In fact, mesenchymal stem cells are presently isolated from bone marrow and then studied for certain purposes or widely used in clinical trials for a variety of diseases.

20 Although it is easy to obtain mesenchymal stem cells from bone marrow, there are difficulties in acquiring bone marrow. Further, it is also difficult to solve the problems associated with an immune rejection reaction occurring when implanting stem cells to others.

25 Meanwhile, it is relatively easy to obtain umbilical cord blood compared with obtaining bone marrow, and also, where great numbers of umbilical cord blood units are secured, it is possible to employ umbilical cord blood stem cells that are identical with or most similar to histocompatibility genes of patients and thereby it is possible to solve the problems associated with immune rejection. However, it is relatively difficult to obtain mesenchymal stem cells from umbilical cord blood, compared with obtaining them from bone marrow and thereby there is difficulty in study and clinical applications.

30 Conventionally, there has been largely used a method of separating and culturing mesenchymal stem cells from umbilical cord blood within 24 hours from birth, using a density gradient centrifugation method. However, when the

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density gradient centrifugation method is applied to cryopreserved umbilical cord blood, it is difficult to separate cells and easy to lose cells, which makes it more difficult to culture mesenchymal stem cells that are present in umbilical cord blood in a minute amounts.

5 As conventional methods of isolating and culturing mesenchymal stem cells, reference is made to US Patent Nos. 5,197,985 and 5,486,359, which disclose a method of proliferating mesenchymal stem cells in isolating and culturing mesenchymal stem cells from human bone marrow. That is, US
10 Patent No. 5,197,985 is directed to a method for inducing human bone marrow-derived mesenchymal stem cells to differentiate into bone-forming cells, comprising: providing human bone marrow-derived mesenchymal stem cells that have been isolated, purified and culturally expanded from a bone marrow specimen by adding the bone marrow specimen to a medium which contains factors which stimulate mesenchymal cell growth without differentiation and
15 allows, when cultured, for selective adherence of only the mesenchymal stem cells to a substrate surface; applying the isolated, purified and culturally expanded human bone marrow-derived mesenchymal stem cells to a porous carrier; and, implanting the porous carrier containing the culturally expanded human bone marrow-derived mesenchymal stem cells into an environment containing factors
20 necessary for differentiating the human mesenchymal stem cells into bone cells. In the method, the porous carrier comprises hydroxyapatite and tricalcium phosphate and the medium is comprised of BGJ_b medium with fetal bovine serum (FBS) or is comprised of F-12 Nutrient Mixture. Further, US Patent No. 5,486,359 discloses isolated human mesenchymal stem cells which can
25 differentiate into cells of more than one tissue type (for example, bone, cartilage, muscle or marrow stroma), a method for isolating, purifying and culturally expanding human mesenchymal stem cells, and characterization and uses thereof, particularly research reagent, diagnostic and therapeutical uses of such cells. In the patent, mesenchymal stem cells are derived from bone marrow and cultured in
30 BGJ_b medium containing fetal bovine serum.

Disclosure of Invention

Technical Problem

Therefore, the present invention has been made in view of the above problems raised in culturing mesenchymal stem cells, and it is an object of the present invention to provide a reproducible method of isolating and culturing mesenchymal stem cells, in which mesenchymal stem cells can be obtained from
5 umbilical cord blood cryopreserved at -196°C without the loss of cells in the course of density gradient centrifugation.

Technical Solution

10 In accordance with an aspect of the present invention, there is provided a method of isolating and culturing mesenchymal stem cells from cryopreserved umbilical cord blood, comprising the steps of:

thawing cryopreserved umbilical cord blood and adding α MEM (alpha-minimum essential medium) thereto, followed by centrifugation to harvest
15 monocytes;

isolating CD133 positive cells from the obtained monocytes; and
subjecting the isolated cells into suspension culture in the α MEM containing Stem Cell Factor, GM-CSF (granulocyte-macrophage colony-stimulating factor), G-CSF (granulocyte colony-stimulating factor), IL-3
20 (interleukin-3) and IL-6 (interleukin-6).

The present invention provides a reproducible method of isolating and culturing mesenchymal stem cells from umbilical cord blood units cryopreserved at -196°C . That is, it is intended to contribute to treatment of intractable diseases using umbilical cord blood, by finding out optimum
25 conditions for isolating and culturing mesenchymal stem cells from umbilical cord blood relatively lacking the number of cells.

In order to achieve this purpose, the present invention is completed by obtaining monocytes from cryopreserved umbilical cord blood, isolating CD133 positive cells, and then culturing the cells in α MEM including Stem Cell Factor, GM-CSF, G-CSF, IL-3 and IL-6, thereby making it possible to secure primitive
30 mesenchymal stem cells and improve the success rate of cell culture. It is difficult to isolate monocytes from cryopreserved umbilical cord blood that contains red blood cells mixed with monocytes. Therefore, it is necessary to select and culture CD133 positive cells that is presumed to contain stem cells.
35 According to the process of the present invention, the success rate of cell culture

is up to about 90%. No case is reported that stem cells are cultured from cryopreserved umbilical cord blood or bone marrow in the conventional method.

Description of the Drawings

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The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

10 Figs. 1 through 6, respectively, are photographs (X 100) showing results after culturing mesenchymal stem cells derived from cryopreserved umbilical cord blood for 5, 7, 10, 14, 20 and 25 days, in accordance with the method of the present invention.

Best Mode

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Hereinafter, a method of isolating and culturing mesenchymal stem cells from cryopreserved umbilical cord blood in accordance with the present invention will be described in more detail with reference to the following Examples. These examples are provided only for illustrating the present invention and should
20 not be construed as limiting the scope and spirit of the present invention.

EXAMPLES

Example 1: Isolation and culture of mesenchymal stem cells

25 Umbilical cord blood units cryopreserved at -196°C was thawed in water bath at 37°C . In order to isolate monocytes from the umbilical cord blood, the umbilical cord blood was diluted with two-fold volume of αMEM (alpha-minimum essential medium, Jeil Biotech Services, Korea) and was centrifuged at 300xg for 10 minutes at room temperature. The separated buffy
30 coat layer was collected, diluted again with two-fold volume of αMEM , overlapped on Ficoll-Hypaque and centrifuged at 300xg for 30 minutes at room temperature.

In separating monocytes from blood, Ficoll-Hypaque, which is a polymer of Ficoll (sucrose polymer) and Hypaque (sodium ditrizoate), is largely used.
35 Ficoll-Hypaque has a specific gravity of 1.077 g/ml, which is heavier than

monocytes, but lighter than red blood cells, which makes it possible to separate the cells from each other by specific gravity difference therebetween. That is, when blood is placed on Ficoll-Hypaque and centrifuged, monocytes are gathered on Ficoll-Hypaque.

5 Monocytes obtained by density gradient centrifugation method were washed twice with a washing α MEM in which additives were not included.

From the obtained monocytes, CD133 positive cells were selected with Isolation kit (Miltenyi Biotec, Germany) as follows: monocytes were added with 100 μ l of blocking reagent to remove non-specific bonding, and then mixed with 10 100 μ l of CD133/Microbead. The mixture was cultured at 4 $^{\circ}$ C for 30 minutes.

The culture was added with ten-fold volume of PBS (D-phosphate buffered saline, Jeil Biotech Services, Korea), centrifuged at 300xg for 10 minutes, and thereafter, PBS was discarded to obtain the cells adhered to the tube. The cells were resuspended with 500 μ l of PBS. After the column of Isolation kit was 15 washed with 3 Ml of PBS, the resuspended cells were loaded and maintained in the column more than 15 minutes. The column, after rinsed with PBS four times, was removed from the kit and then added with PBS in a tube, followed by flushed with plunger to select positive cells.

Next, 20% fetal bovine serum (FBS, Jeil Biotech Services, Korea), Stem 20 Cell Factor (50 ng/Ml), GM-CSF (granulocyte-macrophage colony-stimulating factor; 10 ng/Ml), G-CSF (granulocyte colony-stimulating factor; 10 ng/Ml), IL-3 (interleukin-3; 10 ng/Ml) and IL-6 (interleukin-6; 10 ng/Ml) were added to α MEM containing antibiotics (1000 U/Ml of penicillin G, 1000 μ g/Ml of streptomycin sulfate, Gibco-BRL), an anti-fungal agent (0.25 μ g/Ml amphotericin B) and 2 25 mM of Glutamine (Sigma) and the selected cells were suspended in the concentration of $1 \times 10^6/\text{cm}^2$.

After five-days culturing, suspending cells were removed from the

cultured cell group. When adherent cells were secured, they were cultured for 25 days in α MEM containing 20% fetal bovine serum and antibiotics, with complete replacement of culture medium at intervals of 2 days without washing process.

5 Figs. 1 through 6, respectively, are photographs (X 100) showing results after culturing mesenchymal stem cells derived from cryopreserved umbilical cord blood for 5, 7, 10, 14, 20 and 25 days, in accordance with the method of the present invention. As shown in the figures, when monocytes isolated from umbilical cord blood were cultured, cells adhered to and growing at the bottom of the flask were observed 5 days after culturing and on the 7th day, cells formed colonies and then grown to cells having various shapes. 10 days after culturing, monocytes undergoes differentiation into spindle-shaped cells, and 25 days after culturing, these spindle-shaped cells become complete mesenchymal stem cells through cell division and multiplication, thereby completing culturing.

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Example 2: Characterization of cell surface antigen of cultured mesenchymal stem cells

In order to determine the characteristics of cell surface antigen of the spindle-shaped mesenchymal stem cells isolated and cultured as described above, the cell surface antigen was analyzed by FACS. The results are shown in Table 1 below. FACS (fluorescence activated cell sorting; a flow cytometer) is used to analyze the characteristics of cells by attaching a luminescent immune antigen indicator to the surface thereof, or to separate cells containing a certain antigen indicator depending.

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Table 1

Indicator	Reaction
CD14	-
CD34	-
CD45	-
SH2	+
SH3	+
CD29	+
CD44	+
CD90	+

CD166	(+)
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As shown in Table 1, the stem cells isolated and cultured in accordance with the present invention show negative reaction in CD34, CD45 and CD14, which are characteristic indicator of hematopoietic stem cells, positive reaction in SH2, SH3, CD29 and CD44, which are characteristic indicator of mesenchymal stem cells, and weak positive reaction in CD166. Accordingly, it is confirmed that the cells isolated and cultured in accordance with the present invention are mesenchymal stem cells.

Example 3: Comparison of success rate of mesenchymal stem cell culture

50 units of cryopreserved umbilical cord blood were cultured according to a conventional method and the method of the present invention, and success rates of cell culture were compared therebetween. The results are shown in Table 2 below.

Table 2

	Conventional method	Inventive method
Number of mesenchymal stem cell units acquired	0	49
Success rate of culturing (%)	0	98

As shown in Table 2, the success rate of mesenchymal stem cell culture of the conventional method is 0%, while that of the present invention is a high success rate of 98%.

Industrial Applicability

As described above, in accordance with the present invention, it is possible to effectively isolate and culture mesenchymal stem cells from umbilical cord blood relatively lacking the number of cells, and therefore, umbilical cord blood which is wastefully disposed may be utilized as important means for treating a variety of intractable diseases.

Although the preferred embodiments of the present invention have been

disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.